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The expression and localization of neuregulin-1 (Nrg1) in the gastrointestinal system of the rhesus monkey

Wei-Jiang Zhao

Center for Neuroscience, Shantou University Medical College, People's Republic of China

Abstract: Although Neuregulin-1 (Nrg1) and its cognate receptors have been found at the mRNA level in human gastrointestinal (GI) tract and their functional roles have been evaluated *in vitro*, their morphological distribution in higher mammals are not fully elucidated. The present research focused on morphological distribution of Nrg1 and its receptors, ErbB2 and ErbB4, in main GI tissues of the non-human primate rhesus monkey. The morphological expression of Nrg1 and its ErbB2 and ErbB4 receptors as well as their potential co-localization were determined by double immunofluorescence staining in esophagus, stomach, small intestine and colon tissues derived from the rhesus monkey tissue microarray. The Nrg1 level on each sample was indexed by the fold of integrated fluorescence intensity (IFI) relative to that of one brain cortical tissue from the rhesus monkey. Differential expression of Nrg1 and its receptors ErbB2 and ErbB4 was found in the GI structures, with higher expression levels detected in stomach and small intestine. Co-localization of Nrg1 with ErbB2 and/or ErbB4 receptors was most apparently detected in the stomach, followed by small intestine, colon, and esophagus. This investigation morphologically profiles the differential expression of Nrg1 and its receptors in main GI structures, suggesting an autocrine or paracrine loop-directed Nrg1/ErbB receptor signaling pathway in these organs of higher mammals. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 1, 38–44)*

Key words: Neuregulin-1 (Nrg1); ErbB2, ErbB4; rhesus monkey; GI tract; tissue microarray

Introduction

Neuregulin-1 (Nrg1) is one of the most active members of the epidermal growth factor (EGF)-like family [1]. As a result of the alternative splicing of NRG1 gene, at least six isoforms of Nrg1, including type I to III Nrg1α and Nrg1β, have been identified [2]. In the developing brain Nrg1 was observed in the gray matter of cortex, hypothalamus and cerebellum. In the adult brain, expression of Nrg1 was observed in more extensive brain areas, including the hypothalamus, hippocampus, basal ganglia and brain stem [3, 4]. Interaction of Nrg1 with the dimers of its receptors, including ErbB2, ErbB3, and ErbB4, results in many biological processes [5, 6]. Receptors of Nrg1 were reported to be expressed in the hypothalamic astrocytes, where their activation as a result of paracrine Nrg1 stimulation leads to the secretion of luteinizing hormone-releasing hormone [3, 4]. Recently, Nrg1 was also detected in gonadotroph cells of the anterior pituitary, where it is assumed to mediate prolactin secretion from the lactotrophs in a juxtacrine manner [7, 8]. Nrg1 also promotes the invasive behavior of breast cancer cells *in vitro*, thus enhancing metastatic processes by regulating actin cytoskeleton through autocrine or paracrine mechanism [9, 10]. ErbB receptors and other ErbB ligands including epiregulin, epidermal growth factor (EGF), heparin-binding EGF, transforming growth factor alpha (TGFα) and neuroglycan-C were expressed in gastric and colon cell lines [11]. Similar expression pattern was also demonstrated in colorectal carcinomas, forming receptor/ligand system involved in cancer development and progression as a prognostic indicator [12]. Although a higher expression of epidermal growth factor receptor (EGFR) was associated with increased invasiveness, reduced survival rate and poor

Correspondence address: W.-J. Zhao
Center for Neuroscience, Shantou University Medical College,
22 Xin Ling Road, Shantou, Guandong Province 515041,
P. R. China, tel.: + 86 13794125386; fax: + 86 754 8890 0236
e-mail: neuromancn@yahoo.com.cn for

prognosis in patients with colorectal carcinoma, little is known about the distribution and functions of ErbB2 and ErbB4 in the GI tract [12].

In the rhesus monkey (*Macaca mulatta), in vivo* therapeutic effects of Nrg1 were investigated in multiple disease models [13], such as pacing-induced heart failure [14] in which recombinant Nrg1 increased activity of PKB and Bcl-xl protein and exerted effects on left ventricular volume of healthy *Macaca mulatta*, with no significant effect on cardiac contractility [15]. In addition, widespread expression of ErbB2, ErbB3 and ErbB4 receptor mRNAs throughout telencephalon was observed in juvenile and adult *Macaca mulatta* [16]. However, no morphological expression of Nrg1 was described in the GI system of rhesus monkey.

Since many neuropeptides have been found along the wall of the gastrointestinal (GI) tract we decided to investigate the morphological expression of Nrg1 and its specific ErbB2 and ErbB4 receptors in the major organs of the GI system. The reported findings may increase our understanding of the Nrg1/ErbB receptor signaling-based functions in the GI tract.

Material and methods

Mouse anti-Nrg1α/β antibodies were purchased from Lab Vision (Fremont, CA, USA). Rabbit anti-ErbB2 and ErbB4 antibodies were purchased from Beijing Biosynthesis Biotechnology (Beijing, China). Donkey anti-goat secondary antibody conjugated to Dylight™ 488 and donkey anti-rabbit secondary antibody conjugated to Dylight™ 594 were purchased from the Jackson Laboratory (Jackson Labs, Bar Harbor, ME, USA). The Macaca mulatta tissue microarray was obtained from Chaoying Biotechnology (RhFDA1, Xi'an, Shaanxi, China).

Paraffin embedded 4 μm-thick tissue sections of the major GI organs were dewaxed, and antigen retrieval was performed using 10 mM citrate buffer (pH 6.0 in a 99°C water bath for 40 min. Samples were blocked with 10% normal donkey serum (NDS) and incubated overnight at 4°C with the following primary antibodies: mixture containing mouse monoclonal anti-Nrg1 α/β (1:100) and rat anti-ErbB2 or rat anti-ErbB4 antibodies (1:100). After being washed, samples were incubated at room temperature with donkey anti-mouse antibody conjugated to Dylight™ 488 and donkey anti-rabbit antibody conjugated to Dylight™ 594 (1:500 for both). Nuclei were counterstained with DAPI. Images were acquired using a Zeiss Microscopy system (Axio Imager Z1, Zeiss, Germany). DAPI was excited at 405 nm, Dylight™ 488 at 488 nm, and Dylight™ 594 at 594 nm in a multi-track configuration. In addition, one dewaxed tissue microarray was subjected to hematoxylin and eosin (H&E) staining for basic morphological observations.

Integrated fluorescence intensity (IFI) was used to evaluate the protein levels of Nrg1 on the GI tissue points. The IFI for Nrg1 at each tissue point was obtained using the MultiImage™ Light Cabinet CY3 of the FluorChem HD2 gel imaging system (Alpha Innotech, CA, USA), and the IFI indexed by the optical density was analyzed using Image Tool II software (University of Texas Health Science Center, San Antonio, TX, USA). The IFI was evaluated on the basis of a gray scale ranging from 0-255, and was defined as the fold of the IFI of each GI structure relative to that of the brain cortical tissue.

Results are expressed as means ± SD based on data obtained from at least 3 samples per group. Data were analyzed by independent sample *t* tests and $p < 0.05$ was considered statistically significant [17, 18].

Results

To evaluate the protein levels of Nrg1 in different structures of the GI tract, integrated fluorescence intensity (IFI) of Nrg1 signals were measured as described in Methods. The relative expression was determined by comparing the optic intensity with that of the randomly selected brain cortex as a baseline control. The relative protein presence of Nrg1 in the esophagus, stomach, small intestine and colon tissues were 0.32 ± 0.09, 1.26 ± 0.28, 1.37 ± 0.14 and 0.66 ± 0.12, respectively (Figure 1). Relative Nrg1 IFI in the stomach and small intestine was significantly higher than those in the esophagus ($p < 0.01$ for both) and in the colon tissues (both $p < 0.05$). Relative Nrg1 IFI in the colon tissues was also significantly higher than that in the esophagus ($p < 0.05$). No significant difference was found in the relative Nrg1 IFI between stomach and small intestine.

Basic esophagus structure based on H&E staining was shown in Figure 2A. In the wall of esophagus, Nrg1 (Figure 2C) and ErbB4 (Figure 2D) were found to be localized in the stratified squamous epithelial (SSE) cells bordering the lamina propria (LP). In addition, the two molecules were partially localized there, and their staining in other esophagus structure was relatively weak (Figure 2E, F). In contrast, we were unable to detect presence of the ErbB2 receptor (data not shown).

Basic gastric structure based on H&E staining was shown in Figure 3A. In the stomach, Nrg1 was mainly observed in both the parietal cells predominating in the mid and upper regions of the glands (Figure 3C, G), where both ErbB2 (Figure 3D) and ErbB4 (Figure 3H) receptors were expressed and co-localized with Nrg1 individually (Figure 3E, I). In contrast, the chief cells predominating in the lower region of the gland only expressed Nrg1 ligand (Figure 2B, G).

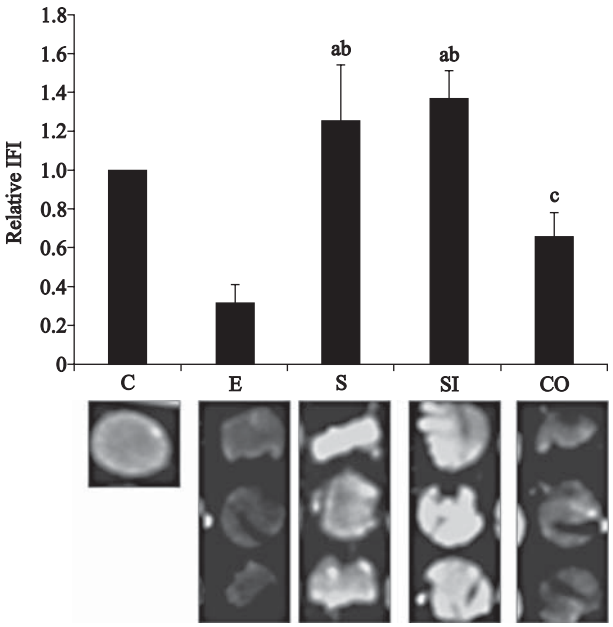

Figure 1. Comparison of Nrg1 integrated immunofluorescence intensity (IFI) in main gastrointestinal tract. Abbreviations. C — control, E — esophagus, S — stomach, SI — small intestine, CO 0 — colon.
[a]$p < 0.01$ compared to values in the esophagus group; [b]$p < 0.05$ compared to values in the colon group; [c]$p < 0.05$ compared to values in the esophagus group

Basic morphology of the small intestine based on the H&E staining was shown in Figure 4A. In the small intestine samples, Nrg1 was selectively expressed in a small population of cells in the lamina propria (Figure 4C, G), whereas ErbB2 was barely detected (Figure 4D, E). In contrast, ErbB4 could be observed in similar cells (Figure 4H), where it co-localized with Nrg1 (Figure 4I). These cells were in morphology and localization similar to the enteroendocrine (EE) cells, which were also indicated in the inset in Figure 4A.

Basic morphology of the colon tissue based on H&E staining was shown in Figure 5A. In the colon, Nrg1 was selectively and weakly localized on the membrane of the absorptive columnar cells (Figure 5C, G), whereas ErbB2 (Figure 5D) and ErbB4 (Figure 5H) were almost undetectable. And no co-localization of Nrg1 and ErbB2 (Figure 5E) and ErbB4 (Figure 5I) was found.

Discussion

Although expression, localization and functions of Nrg1 and its receptors were described in various tissues and organs, their morphological distribution and localization in the main parts of the GI tract of the *Macaca mulatta* have not been well elucidated. This prompted us to investigate the expression and localization of Nrg1 and ErbB2/ErbB4 in the GI tissues of this non-human primate. The main findings showed differential expression and distribution of Nrg1 and its cognate receptors in the GI tract, with higher expression of Nrg1, ErbB4 and/or ErbB2 in the stomach and the small intestine.

In one study of the human upper GI mucosa biopsy specimen, type I Nrg1, ErbB3 and ErbB4 mRNA were detected in esophagus, stomach and duodenum with the highest expression found in duodenum [19]. These findings suggested the physiological significance of Nrg1 and its receptors in the human upper GI mucosa. However, cells expressing Nrg1 and ErbB receptors were not further identified. Compared with RT-PCR, immunofluorescence-based investigation

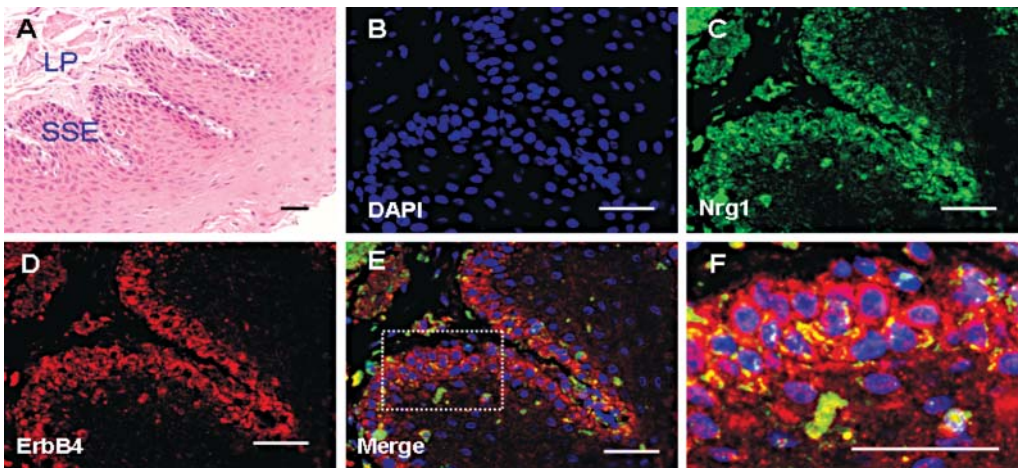

Figure 2. Expression and localization of Nrg1 and ErbB4 in the Rhesus monkey esophagus tissue. **A.** H&E staining; **B.** DAPI staining of cell nuclei (blue); **C.** expression of Nrg1 (green); **D.** expression of Erb4 (red); **E.** co-localization of Nrg1 and Erb4 (yellow); **F.** magnified insert from E. SSE: stratified squamous epithelium; LP: lamina propria. Scale bars = 50 μm

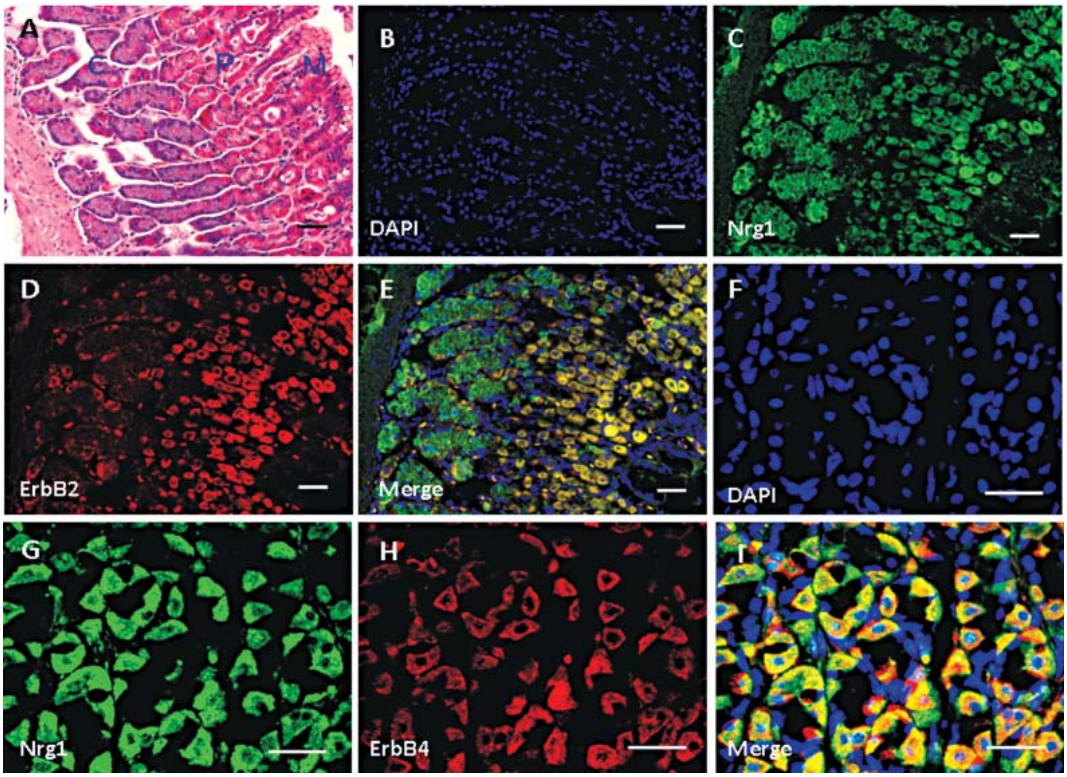

Figure 3. Expression and localization of Nrg1, as well as ErbB2/ErbB4 in the Rhesus monkey gastric tissue. **A.** H&E staining; **B and F.** DAPI staining of cell nuclei (blue); **C and G.** expression of Nrg1 (green); **D.** expression of ErbB2 (red); **E.** co-localization of Nrg1 and Erb2 (yellow); **H.** expression of ErbB4 (red); **I.** co-localization of Nrg1 and Erb4 (yellow). M: mucosa; PC: parietal cell; CC: chief cell. Scale bars = 50 μm

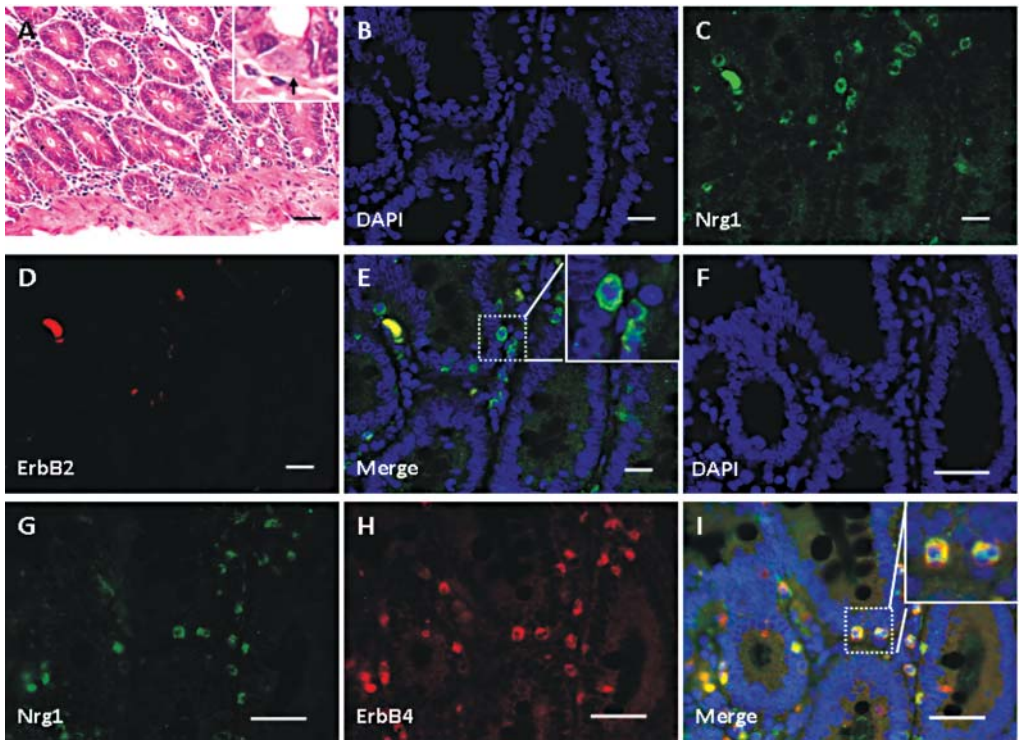

Figure 4. Expression and localization of Nrg1, as well as ErbB2/ErbB4 in the Rhesus monkey small intestinal tissue. **A.** H&E staining; **B and F.** DAPI staining of cell nuclei (blue); **C and G.** expression of Nrg1 (green); **D.** expression of ErbB2 (red); **E.** co-localization of Nrg1 and Erb2 (yellow); **H.** expression of ErbB4 (red); **I.** co-localization of Nrg1 and Erb4 (yellow). The arrow indicates a cell, which is morphologically similar to the enteroendocrine (EE) cell. Scale bars = 50 μm

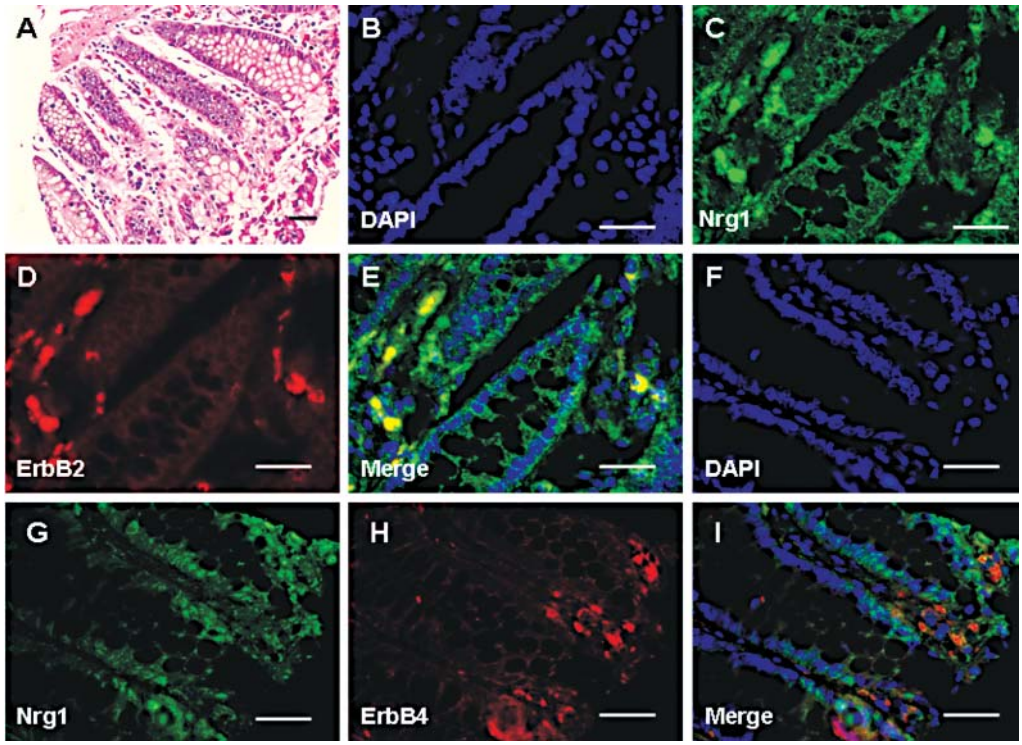

Figure 5. Expression and localization of Nrg1, as well as ErbB2/ErbB4 in the Rhesus monkey colon tissue. **A.** H&E staining; **B and F.** DAPI staining of cell nuclei (blue); C and G, Nrg1 was found in a main population of the absorptive columnar cells (green); **D.** expression of ErbB2 (red); **E.** co-localization of Nrg1 and Erb2 was undetectable; **H.** expression of ErbB4 (red); **I.** co-localization of Nrg1 and Erb4 was not detected. Scale bars = 50 μm

can clarify in detail the distribution of Nrg1 and ErbB receptors and types of cells expressing them. Here, Nrg1 and ErbB2/ErbB4 receptors were sparsely and weakly expressed in the esophagus and colon tissues, which may suggest that they are not functionally vital in these tissues. However, both common and rare variants of the NRG1 gene have been reported to contribute to Hirschsprung disease (HSCR, congenital colon aganglionosis), a relatively common complex genetic condition caused by abnormal development of the enteric nervous system (ENS) [20].

In the investigated GI tissues, the highest level of Nrg1 was observed in the stomach and the small intestine, suggesting that physiological autocrine/juxtacrine interaction of Nrg1 with their receptors may exert specific, even if unknown, functions in the upper GI tract. In contrast to the low level in the small intestine tissue, the staining intensity of ErbB2 was the same to that of ErbB4 receptor in parietal cells which predominated in the mid and upper regions of gastric and small intestinal glands. These observations may suggest possibility of the formation of ErbB2/ /ErbB4 heterodimeric complex and their involvement in the autocrine signaling initiated by Nrg1 in the gastric structures, while the ErbB4/ErbB4 homodimeric complex may predominately exist in the small intestine tissue. Nrg1-stimulated heterodimerization represents a key feature of mitogenic signaling in mammary epithelial cells and tumors, including cell proliferation, differentiation and migration, and malignant transformation [21, 22]. It was found that in various cells ErbB2 can preferentially form heterodimers with both EGFR, as well as with ErbB3 and ErbB4 [23, 24].

The balance of ErbB4/ErbB4 homodimers versus its heterodimeric complexes with other EGFR family members determines cell proliferation or differentiation. Introduction of ErbB4 in ErbB4-negative SUM102 cells resulted in cell differentiation and was both necessary and sufficient to trigger an antiproliferative response in human breast cancer cells [24]. Recently, somatic mutations altering the coding region of ErbB4 were described in patients with gastric and colorectal cancer [25]. Considering the hydrochloric acid secretion function of the parietal cells, we hypothesize that the abundant expression of Nrg1 and ErbB2/ErbB4 may function by protecting the self-damage of cells by hydrochloric acid and may modulate the parietal cell differentiation and renewal. Nrg1 ligation to ErbB4 promotes its heterodimerization

with ErbB2, and consequent ErbB2 tyrosine phosphorylation, thus affecting colon cancer growth [26]. It has been reported that ErbB2 and ErbB3, but not ErbB4, were expressed in gastric cancer cell lines, with their cognate ligand Nrg1α in gastric fibroblasts [27]. Thus, a mesenchymal-epithelium-based paracrine activation of intracellular signal pathway may exist in the gastric mucosa. It was found that EGF can induce cell growth and differentiation in the absence of EGFR to form a heterodimer between ErbB3 and ErbB2, a previously identified oncogenic complex in response to EGF and betacellulin [28]. In some other conditions, activation of ErbB2 was the result of Nrg1-mediated interaction with ErbB3 and it generated downstream activation of the ERK and the phosphatidylinositol 3'-kinase (PI3K)/AKT pathways [29]. However, Tsai et al. [30] reported that blocking Nrg1 expression suppressed the aggressive phenotype of MDA-MB-231 breast cancer cells, as evidenced by inhibited cell proliferation, anchorage-independent growth failure and the suppression of the cell invasion. This suggests that Nrg1 can function independent of ErbB2 overexpression.

Based on the observation about the presence of ErbB4 and the absence of ErbB2 in the cells of the small intestine, it may be concluded that ErbB4 homodimers represent the predominant functional ErbB receptor pattern in this part of the GI tract. Zhao and colleagues [6, 7] reported that Nrg1 can induce PRL secretion from rat lactosomatotroph GH3 cells in either an autocrine or a paracrine manner in vitro, suggesting that Nrg1 may possibly be responsible for hormone production and secretion in the small intestine. Although ErbB4 was absent in the colon tissue, high levels of ErbB4 receptors were observed in human and mouse colitis [31]. Increased ErbB4 expression synergizes with proinflammatory cytokines to upregulate COX-2 expression and to inhibit colon epithelial apoptosis. Such a process may be involved in the development of tumorigenesis [31]. In vitro, Nrg1 acts to stimulate the proliferation and alters the cellular morphology of colonic epithelial cells in culture, with Nrg1β exhibiting more pronounced effects than the alpha isoform [32]. NRG4 and ErbB4 were also found to be mainly expressed in mucosa-associated lymphoid tissue (MALT) and follicular lymphoma, where they function in the proliferation of malignant lymphoma cells in the GI tract [33]. Thus, expression of Nrg1 in the colon may also regulate growth and differentiation of colonic epithelial cells under pathologic conditions.

EGF receptor family members including EGFR and ErbB2 are main candidates for the molecular-targeted therapy of gastric cancer and colon cancer due to their overexpression [34]. Blockade of ErbB2 activation by Nrg1 neutralizing antibodies prevented cell cycle re-entry and reduced Nrg1β1-induced migration and invasion and also induced apoptosis in colon cancer cell lines [29, 34]. Neutralization of Nrg1 function also abolished radiation-induced AKT activation and reverted the radiosensitivity of HCT116 colorectal carcinoma cells to a lower level [35]. In addition, anti-ErbB2 monoclonal antibody 2C4 can block Nrg1-stimulated phosphorylation of ErbB2 and ErbB3, activation of mitogen-activated protein kinase (MAPK), PI3K, and Akt, accompanied with inhibited proliferation and anchorage-independent growth [36].

In conclusion, we observed the preferential expression of Nrg1 with their receptors ErbB4 and/or ErbB2 in the gastric and small intestinal tissues, supporting the autocrine role of Nrg1 in modulating cell differentiation and proliferation through ErbB4/ErbB4 homodimers and ErbB4/ErbB2 heterodimers in these regions. These observations require further studies to explore the role of Nrg1 and its receptors in the pathogenesis of the GI tract diseases.

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